THE CHEMISTRY OF THE INDIVIDUAL NEURON

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MEDICINE is entering a chemical age. It seems proper to say "entering" rather than "has entered", because the things we know about the chemistry of living things must be only a minute fraction of what there is to know. Also, we are still "entering" in the sense that many branches of the life sciences are a little reluctant to retool for their chemical problems. In some branches the inertia has been so great that it has been necessary to invent new names for the chemically oriented recruits. There are "biophysicists" (i.e., physical chemists) in physiology and "molecular biologists" in biology. We already have "molecular medicine" and surely before long will have "molecular biochemistry".

As neurology, along with the rest of medicine, enters the chemical age, it encounters major problems. First, of course, are problems arising from the uniquely complicated structure of the nervous system. No other part of the body has such a variety of cell types arranged with anything approaching the same complexity. Neurons vary a thousandfold in mass and in length. Neuronal and glial cell bodies are intimately mingled and their processes tangle together in a dense felt work. Everything indicates large chemical differences among the various histological elements, so that chemical analyses of gross samples of nervous tissue are scarcely interpretable.

The second difficulty comes from the inherent complexity of the chemical machinery of any living cell. Guesses as to the number of enzymes in a cell range up to 10,000. This orchestra of thousands of members, playing several simultaneous symphonies on many hundreds of different instruments, usually plays in harmony in spite of continual replacement of many of the players. Fortunately, in confronting basic biochemical problems, we have the help of the whole biochemical

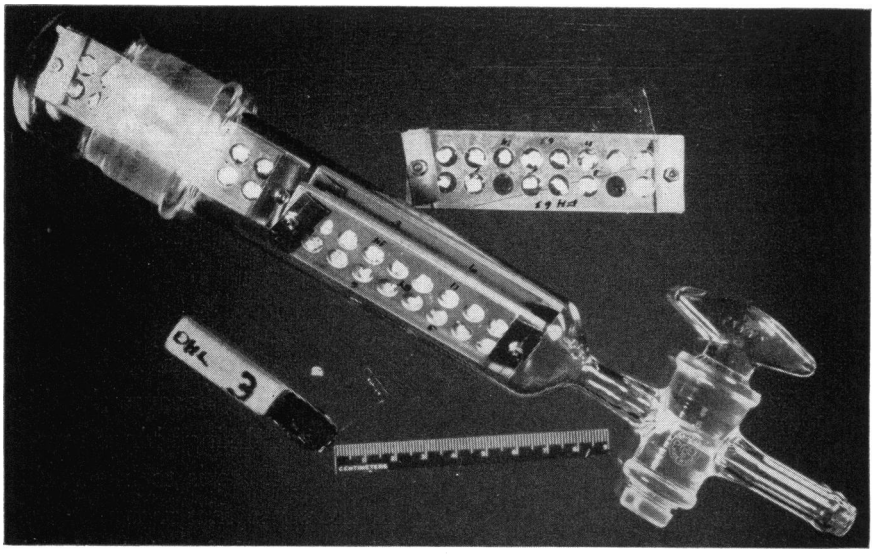

Fig. 1. Aluminum racks for receiving microtome sections. Three of these are shown in a glass tube in which the samples are dried under vacuum at low temperature and in which they are subsequently stored. Also shown is a temporary wooden and glass holder for carrying dissected samples to the balance. Five samples weighing about one microgram are shown.

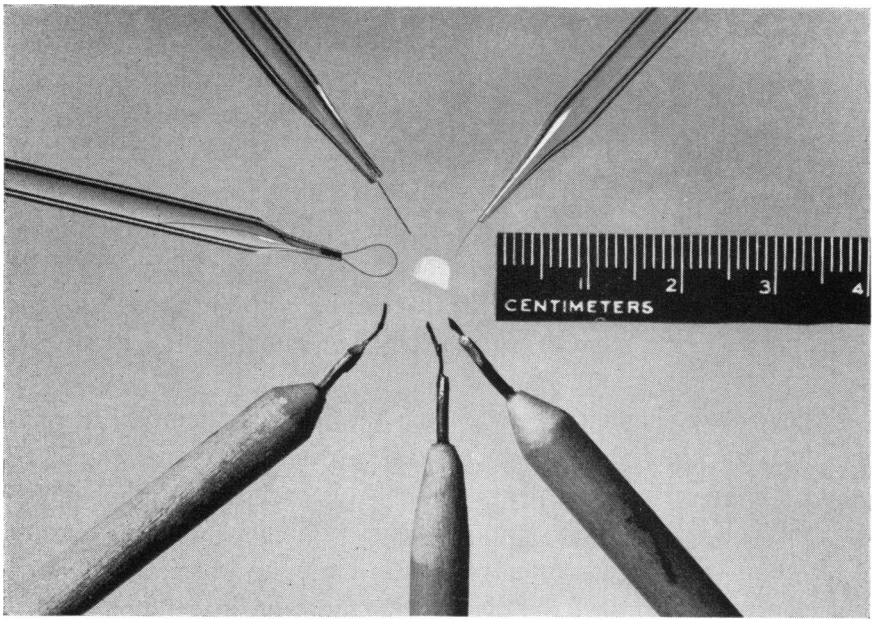

Fig. 2. Knives, hair loop and hair points used in dissection and manipulation of samples. A piece of a frozen dried section of brain tissue is also shown.

world, since most of the general problems are common to all forms of life. Without this help we would be a long time on the road.

The third group of problems grows from the first. They concern the spotty nature of diseases of the nervous system. The microscopist can examine a damaged cell alongside one that has been spared, or can distinguish demyelination from axonal damage, or can see around the macrophage that has come in as a consequence of a disease process; but all of these things confuse the chemical analysis of gross specimens.

My purpose here is to describe a histochemical methodology designed to get around the problems of structural complexity and of the complexity of neuropathological change. The general scheme is based on old-fashioned test tube biochemistry—with perhaps a slight twist.

The first step is designed to isolate samples for analysis without alteration of the substances to be measured.[1] The tissue is frozen quickly in Freon (CH_2F_2) at $-150°C$. In the case of analyses for enzymes, lipids and other fairly stable components there is no particular difficulty. In the case of labile substances such as glucose, phosphocreatine, and many other metabolites, the speed of freezing is very critical and presents special problems that go beyond the purpose of the present discussion.

The frozen specimen is sectioned at $-20°C$ in a cryostat, and the sections are transferred to an aluminum rack sandwiched between two microscope slides (Figure 1). A number of these racks are then transferred to a large glass tube (Figure 1) in which the sections are dried under vacuum at $-40°$.

Most enzymes and substrates in the frozen-dried sections are stable for years at $-20°C$, and for many hours at room temperature, provided the humidity is kept below 50 or 60 per cent. Therefore, the histological structures can be dissected at leisure at room temperature under suitable magnification. For this purpose, knives made from splinters of razor blades are satisfactory (Figure 2). These are sealed to a short piece of wire in a suitable handle, and in some cases a short piece of horse hair is interposed between blade and wire to give flexibility. For dissection, the section is held down with a hair loop, while the knife cuts are made, or else two knives are used as needles to tease out the structure desired. Freehand dissection is most convenient for structures down to 30 to 40 μ in diameter; below this, particularly as the age of the dissector increases, a mechanical micromanipulator becomes necessary. In spite of the fact that the frozen-dried tissue is neither stained nor embedded,

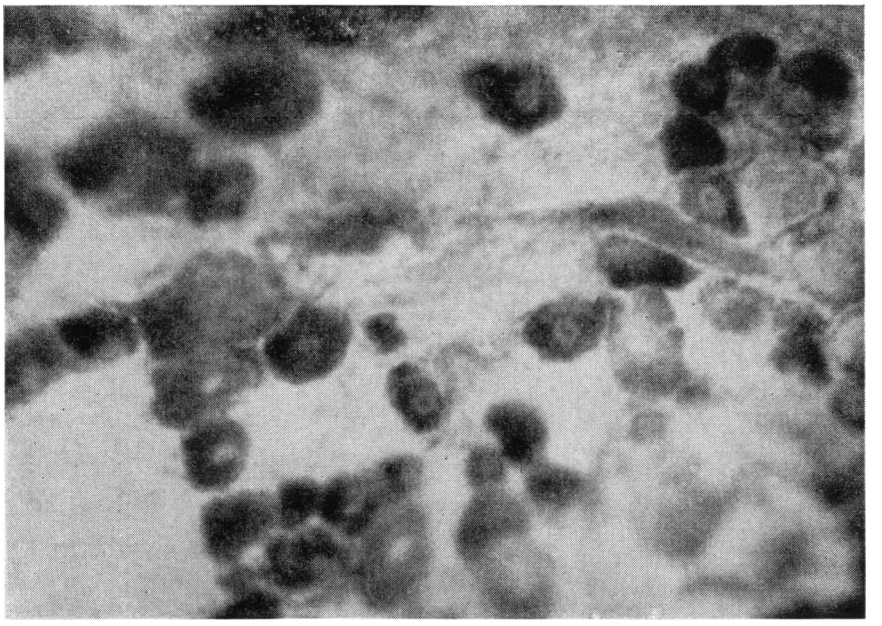

Fig. 3. Frozen dried sample from a dorsal root ganglion of the rabbit. This is unfixed and unstained.

larger structures, at least, are readily visible (Figure 3).

The next step is to measure sample size, and this is most readily accomplished by weighing.[1] The balances used are simply small fish-poles made of quartz fibers mounted in syringes of appropriate size (Figure 4). A series of balances are required to cover the useful range of histochemical analysis. The balance shown is the most sensitive to date. It is capable of weighing samples in the range from 0.000,2 to 0.002 μg. Much more sensitive balances of the same type could probably be constructed.

In planning for analysis of histological elements of the nervous system, consideration is given to the size of sample and the sensitivity of chemical methods that may be available. The largest neuronal cell bodies in the mammal (anterior horn cells, dorsal root ganglion cells) fall in the range of 0.02 μg dry weight. The smallest cell bodies may weigh only a thousandth as much (2×10^{-5} μg). An anterior horn cell body contains enough of an enzyme, such as lactic dehydrogenase, to produce 10^{-9} moles of product in an hour's incubation. Other less abundant enzymes may yield as little as 10^{-13} moles of product in the

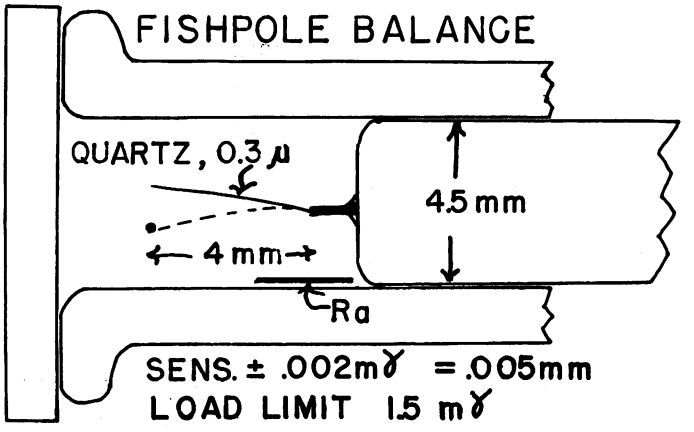

Fig 4. Quartz fiber balance. The radium is introduced into the chamber to prevent possibility of accumulation of static electrical charges. The balance case is made from a cut-off 1 ml. tuberculin syringe with reversed plunger.

same time, whereas the smallest cell bodies would be expected to contain only enough of the least active enzymes to produce 10^{-16} moles of product in an hour. Similiarly, there may be 10^{-13} moles of glucose in an anterior horn cell body, but only 10^{-18} moles of pyruvate in the smallest cell body. Thus, useful information about the more abundant enzymes can be obtained with methods having sensitivity down to 10^{-10} moles, but to cover most enzymes and substrates in structures down to the size of a 5 μ sphere, would require methods capable of measuring 10^{-18} moles of material. This is approximately one trillionth of the amount needed in the conventional Warburg apparatus (10^{-6} moles).

Giacobini has refined the cartesian diver technique of gasometric analysis to a point where it is possible to measure in the 10^{-12} to 10^{-14} mole range,[2] and has applied this tool to the measurement of parts of individual neurons for cholinesterase[3] and carbonic anhydrase.[4]

Colorimetric methods even with microcuvettes are generally useful only down to the range of 10^{-9} moles. Nevertheless, with special optical techniques, colorimetric methods are capable of much greater sensitivity. Ornstein and Lehrer[5] have devised a technique for carrying out colorimetric analysis in minute droplets that promises to have great sensitivity. Edström and coworkers have devised methods for spectroscopic measurement of purine and pyrimidine bases after chromatography on single rayon fibers in amounts as small as 10^{-13} moles.[6]

Fluorimetric methods are inherently something like a thousand

times more sensitive than colorimetric methods. The pyridine nucleotides DPN and TPN can be measured fluorimetrically; and almost any biological material, with the aid of suitable enzymes, can be made to oxidize or reduce a pyridine nucleotide. Because of these three attributes, we have come to rely almost entirely on fluorimetry with pyridine nucleotides as the basic analytical tool.[7, 8]

For example, if the problem is to measure glucose, the sample, with its enzymes suitably inactivated, is added to a small volume of reagent containing ATP, hexokinase, TPN^+ and glucose-6-P dehydrogenase. During a short incubation the glucose is converted to 6-P-gluconate with the formation of an equivalent amount of TPNH. If the initial amount of glucose had been 10^{-10} moles or more, the sample is simply diluted to 1 ml and the TPNH measured by its native fluorescence (the TPN^+, present in excess is not fluorescent). If the amount of glucose had been in the range of 10^{-10} to 10^{-11} moles, the TPNH is measured indirectly by first destroying the TPN^+ with weak alkali (to which TPNH is completely insensitive) following which the sample is treated with peroxide (to oxidize TPNH to TPN^+) in 6-N NaOH. A very strongly fluorescent product is formed.[7, 9] In the measurement of various substances, the products may be TPN^+, TPNH, DPN^+ or DPNH. Both TPNH and DPNH are stable in alkali but unstable in acid, whereas exactly the reverse is true for TPN^+ and DPN^+. Consequently, it is always possible to destroy the unused excess of the starting form of the pyridine nucleotide after completing a given reaction.[10] Subsequently, the pyridine nucleotide produced can be measured fluorimetrically.

Such reaction, as stated, can measure 10^{-11} moles of material (or 10^{-12} moles if microcuvettes are used). But for smaller quantities, more sensitive means had to be devised. This was accomplished by using the pyridine nucleotide produced in the primary reaction to catalyze a two-enzyme system and thereby bring about the formation of a large amount of the enzyme products, one of which could be measured in a third step with great increase in sensitivity.[11]

The system used for TPN^+ or TPNH is the following:

1. $\text{TPNH} + \text{NH}_3 + \alpha\text{-ketoglutarate} \xrightarrow[\text{dehydrogenase}]{\text{glutamate}} \text{TPN}^+ + \text{glutamate}$

2. $\text{TPN}^+ + \text{glucose-6-P} \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-P}} \text{TPNH} + \text{6-P-gluconate}$

These reactions follow each other in a cyclic manner. With suitable amounts of the two enzymes as much as 20,000 moles of the products glutamate and 6-P-gluconate may be produced in an hour's incubation for each mole of TPNH (or TPN^+) present. After such incubation, the reactions are stopped by heating briefly at 100°C, following which the 6-P-gluconate is measured with an excess of TPN^+ which is now added:

$$3.\ TPN^+ + \text{6-P-gluconate} \xrightarrow[\text{dehydrogenase}]{\text{6-P-gluconate}} TPNH + \text{ribulose-5-P} + CO_2$$

Thus the TPNH (or TPN^+) has been amplified as much as 20,000-fold. If necessary the process can be repeated using the TPNH formed in reaction 3 after removing the excess TPN^+ by exposure to alkali. Overall enhancement of 45,000,000 has been attained without sacrifice of precision.[11]

A similar "cycling" method is available for measuring DPN^+ or DPNH. Consequently, analytical sensitivity appears to be available for nearly any biological material down to about 10^{-15} moles with one cycle, and down to 10^{-18} or 10^{-19} moles with double cycling. This means that it is possible, in principle, to measure 10,000 molecules of almost any substrate or a single molecule of almost any enzyme.

Thus, sensitivity *per se* would not seem to be an obstacle to the chemical study of structures of the nervous system of the size of the smallest cell bodies. So far most studies have been made with larger cell bodies. Measurements of a variety of enzymes on dorsal root ganglion cell bodies and anterior horn cell bodies demonstrate that satisfactory precision can be obtained with direct chemical analysis of structures weighing in the order of 0.01 μg.[12, 13]

Robins and coworkers have carried out a series of measurements of changes in single anterior horn cells of the monkey during chromatolysis. These studies demonstrate the practicality of experimental work based on direct analysis of the individual affected neurons.[14]

There are fewer studies to date with smaller structures, but there seem to be no major obstacles. Table I records analyses for hexokinase in nuclei and cytoplasm of dorsal root ganglion cell bodies from the rabbit. These samples weighed about 0.000,5 μg. Although there were a few minor technical additions required in dealing with these smaller samples (Figure 5) no real difficulties were encountered.[15]

Except for the DNA and RNA studies of Edström and coworkers direct quantitative analyses of single neurons have been limited to

TABLE I—HEXOKINASE IN NUCLEI AND CYTOPLASM OF DORSAL ROOT GANGLION CELL BODIES (RABBIT)

Values are recorded as moles of glucose phosphorylated per kg. dry weight per hour. The nuclei weighed from 0.3 to 0.6 millimicrograms. The size of the samples of cytoplasm (from the same cells, as indicated) was in the same range. Four samples of whole brain homogenate, each equivalent to 0.43 millimicrograms dry weight, gave values of 6.2, 6.8, 7.3 and 6.0 moles per kg. per hour.

Cell No.	1	2	3	4	5	6
Nucleus	1.0	0.8	0.8	1.2	1.5	1.6
Cytoplasm	2.0	1.5	2.9	2.3	2.6	2.0
Cytoplasm	2.2	2.4	2.7	1.5	2.1	3.8

Av. of 13 cells:	Nucleus	1.31
	Cytoplasm	2.59
	Difference	1.28 ± .18 (st. error)

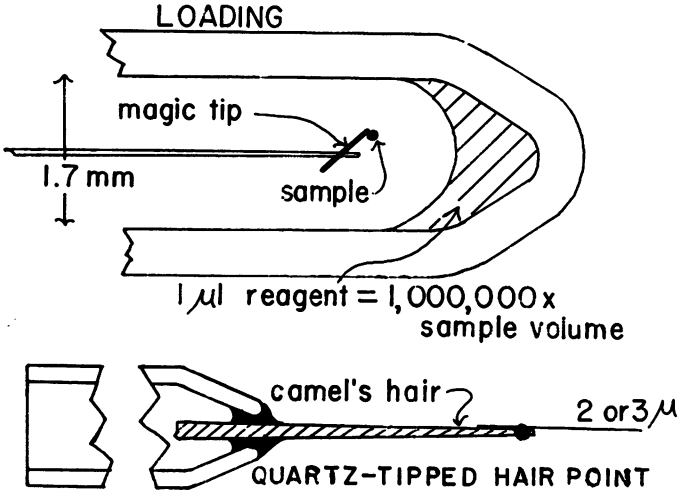

Fig. 5. Arrangement for adding the nucleus of a large nerve cell to reagent for measurement of an enzyme activity. The "magic tip" is a piece of fiber glass 0.3 to 0.5 mm. in length and about 10 μ in diameter. This is placed on the end of a long quartz needle where it is held by surface forces. The sample is transferred to the fiber glass fragment with the quartz-tipped hair point. A micro test tube with reagent is then slid over the sample, with the aid of a rack and pinion, until the "magic tip" and sample are washed off into the reagent.

enzyme measurements. Valid measurements of the concentrations of enzymes present indicate enzymatic *capability* but do not tell how much use is made of these enzymes. Much more information could be obtained from knowledge of the concentrations of the substrates of the enzymes concerned, and the changes in the substrate levels induced by alteration in supply of oxygen, glucose, and by the application of drugs.

Since the original work of Kerr in the 1930's, it has been known that within seconds after shutting off the blood supply to the brain there occur profound decreases in the levels of glucose, P-creatine, and ATP, and an abrupt rise in lactate. On the one hand, this constitutes a difficulty in obtaining true resting values; on the other, it presents an opportunity for study of the metabolism of either the brain as a whole, or of any of its parts. With the blood supply cut off the only major sources of energy consist of ATP, P-creatine and the ATP generated when glucose and glycogen are converted to lactate. From the changes in these five substrates during the first 30 to 60 seconds, while the nervous system still survives, it is possible to calculate the rate of high energy phosphate use (i.e., the metabolic rate). If such measurements were made on anterior horn cell bodies, for example, on a series of animals with tissue frozen at exactly measured time intervals after interrupting the blood supply to the cord, the metabolic rate of these cell bodies could be determined. Furthermore, the relative order of change, and the levels of intermediate substrates during this process would provide intimate details about the functioning of the glycolytic system in whatever structures are analyzed. The effects of drugs or metabolic inhibitors on these changes would provide useful information.

As stated earlier, the measurement of substrates demands greater sensitivity than the measurement of enzyme activities. The cycling methods provide sufficient sensitivity in principle, but there are new technical problems to be overcome. The chief problem is the necessity to keep *concentrations* of the substances to be measured sufficiently high to avoid serious errors from high blank values. This means working with very small volumes. Preliminary studies have shown the advantage of switching from the use of micro test tubes to the performance of the necessary chemical steps in small droplets under oil. Problems of evaporation, exposure to CO_2, and surface contamination become troublesome in test tubes with volumes of 1 μl. or less. Under oil it is

practical to work with volumes of 0.01 μl. and probably much smaller volumes could be used.

In conclusion, I would like to leave the impression that it is easy to perform direct chemical analyses of tissue samples down to very small sizes, but I would not want to create the impression that chemistry itself is easy. I do not forsee any great difficulty in measuring almost anything in structures as small as those easily seen under the light microscope. But chemistry of living things—at any scale—is terribly complex. I believe that it is not too complex to comprehend some day, but it will take time, and it will take many people combining their efforts and using many kinds of tools.

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